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SUBJECT AREAS:

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MICROBIOLOGY

Laccase production by *Corioloopsis caperata* RCK2011: Optimization under solid state fermentation by Taguchi DOE methodology

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Laccase production by *Corioloopsis caperata* RCK2011 under solid state fermentation was optimized following Taguchi design of experiment. An orthogonal array layout of $L_{18} (2^1 \times 3^7)$ was constructed using Qualitek-4 software with eight most influential factors on laccase production. At individual level pH contributed higher influence, whereas, corn steep liquor (CSL) accounted for more than 50% of the severity index with biotin and KH_2PO_4 at the interactive level. The optimum conditions derived were; temperature 30°C , pH 5.0, wheat bran 5.0 g, inoculum size 0.5 ml (fungal cell mass = 0.015 g dry wt.), biotin 0.5% w/v, KH_2PO_4 0.013% w/v, CSL 0.1% v/v and 0.5 mM xylinine as an inducer. The validation experiments using optimized conditions confirmed an improvement in enzyme production by 58.01%. The laccase production to the level of 1623.55 Ugd^{-1} indicates that the fungus *C. caperata* RCK2011 has the commercial potential for laccase.

Laccase (EC 1.10.3.2; benzenediol: oxygen oxidoreductase), a multicopper enzyme belonging to the blue oxidases, was first detected in 1883 in the exudates of the Japanese lacquer tree, *Rhus vernicifera*¹. Majority of the laccases have been reported from white rot fungi, however, they could be found in some other type of fungi, bacteria and insects². These enzymes are characterized by their ability to catalyze one-electron oxidation of four reducing-substrate molecules concomitant with four-electron reduction of molecular oxygen to water. Currently the catalytic properties of laccases are being exploited and they have become industrially important enzymes because of their diverse applications: in pulp bleaching, detergents, adhesives, fibre functionalization, detoxification, denim bleaching, textile dye decolorization, baking, biosensors and in biofuel cells^{3,4}.

Laccase production from microorganisms has been reported under both submerged fermentation (SmF) and solid state fermentation (SSF) conditions. SSF, whereby an insoluble substrate is fermented with sufficient but no free moisture, typically uses agricultural residues such as wheat bran, wheat straw, rice bran etc., and offers numerous economical and practical advantages over SmF. Laccase production under different fermentation conditions is influenced by physical and chemical environment of cultural conditions, such as quality and quantity of carbon and nitrogen source, pH, temperature, aeration, presence of inducers, vitamins, amino acids, phosphorus, metal ion etc.⁵⁻⁷. For effective laccase production, it is essential to optimize simultaneously the culture conditions and composition of media. Therefore, the optimum conditions for the laccase production should be investigated for a cost effective commercial production. In the conventional approach, an optimization process usually involves one factor at a time (OFAT). This procedure is time consuming, cumbersome, requires more experimental data sets and do not provide information about the mutual interactions of the parameters. In contrast, statistically planned experiments reduce the number of experiments by developing a specific design of experiments, which also minimize the error in determining the values for significant parameters. Therefore, statistical tools and related experimental design helps to gain more information about the optimization conditions. In this regards, normally the response surface methodology (RSM) has received much attention in scientific researches⁸.

Recently Taguchi method, another method for designing factorial experiments, has been used by some researchers⁹, possesses some advantages, such as much quantitative information can be extracted by only a



Serial No.	Factor	Level 1	Level 2	Level 3
1	Temperature (°C)	30	35	-
2	pH	4.5	5.0	5.5
3	Wheat bran (g)	3	5	7
4	Inoculum (ml)	0.5	1.0	1.5
5	Biotin (% w/v)	0.4	0.5	0.6
6	KH ₂ PO ₄ (% w/v)	0.03	0.05	0.07
7	Corn steep liquor (% v/v)	0.1	0.2	0.3
8	Xylidine (inducer) (mM)	0.5	1.0	1.5

few experiment trials and provides a systematic and efficient plan for performing experiments under the consideration of the interactive effects among the control factors¹⁰. Taguchi method is one of the powerful optimization techniques and requires half of the time than RSM^{11,12}. Although, Taguchi method has gained much popularity in engineering applications, its utilization in the field of biotechnology has been somewhat limited. However, recent studies have confirmed its applicability to optimize biochemical processes. It was successfully employed to improve xylanase production by 227%¹³ and to increase L-asparaginase production by *Staphylococcus* isolate¹⁴. Taguchi method of orthogonal array (OA) design of experiment (DOE) is a simple statistical tool to obtain extensive parameter data from only a few experiments. It involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels)^{15,16}. Unlike traditional DOE, which focuses on the average process performance characteristics, it concentrates on the effect of variation on the process characteristics¹⁷ and makes the product or process performance insensitive to variation by proper design of parameters. This approach also facilitates to identify the influence of individual factors, establish relationship between variables and operational conditions and finally establish the performance at the optimum levels obtained with a few well-defined experimental sets¹⁸. Analysis of variance (ANOVA) was applied to determine optimum configuration of optimized variables. This approach does not only help in saving considerable time and cost but also leads to a more fully developed process by providing systematic, simple and efficient methodology for the optimization of the near optimum design parameters with only a few well defined experimental sets¹⁹.

In the present study, the objective was to identify the best conditions for the production of extracellular laccase by *Corioliopsis caperata* RCK2011 under SSF by applying Taguchi DOE. The experiments were designed with eight factors including temperature, pH, wheat bran, inoculum size, corn steep liquor (CSL), KH₂PO₄, biotin and xylidine for laccase production at three levels with OA layout of L₁₈ (2¹ × 3⁷).

Results

Evaluation of bioprocess for laccase production. Optimization of culture conditions by preliminary studies indicated that medium temperature - 30°C, pH - 5.0, wheat bran - 5 g, inoculum size - 1 ml (0.015 g dry wt.), nitrogen source (NH₄Cl - 0.15% N₂ equivalent), MgSO₄ - 0.1% (w/v), KH₂PO₄ - 0.05% (w/v), CSL - 0.2% (v/v), vitamins (biotin) - 0.5% (w/v) and inducer (xylidine) - 1.0 mM are critical fermentation factors for laccase production by *C. caperata* RCK2011 (results not shown). Under these optimized conditions the production of laccase was improved by 77% (from 211.45 Ugds⁻¹ to 916.0 Ugds⁻¹). The results suggested that optimization of each fermentation factor such as temperature, pH, carbon, nitrogen, vitamins, inducers and others are the prerequisite for laccase production at an industrial scale. However, the conventional method of optimization does not provide any information about interactive influence of the factors responsible for higher production, which is known to positively regulate the

Experiment No.	Column								Laccase Activity (Ugds ⁻¹) ± S.D.
	1	2	3	4	5	6	7	8	
1	1	1	1	1	1	1	1	1	1576.13 ± 4.75
2	1	1	2	2	2	2	2	2	1330.46 ± 61.18
3	1	1	3	3	3	3	3	3	667.66 ± 5.57
4	1	2	1	1	2	2	3	3	1053.48 ± 48.88
5	1	2	2	2	3	3	1	1	1075.54 ± 10.79
6	1	2	3	3	1	1	2	2	830.08 ± 11.34
7	1	3	1	2	1	3	2	3	206.26 ± 9.62
8	1	3	2	3	2	1	3	1	759.86 ± 38.69
9	1	3	3	1	3	2	1	2	259.13 ± 1.06
10	2	1	1	3	3	2	2	1	310.35 ± 6.15
11	2	1	2	1	1	3	3	2	783.21 ± 21.62
12	2	1	3	2	2	1	1	3	524.93 ± 5.33
13	2	2	1	2	3	1	3	2	643.64 ± 18.12
14	2	2	2	3	1	2	1	3	730.47 ± 75.31
15	2	2	3	1	2	3	2	1	952.21 ± 26.91
16	2	3	1	3	2	3	1	2	49.23 ± 1.40
17	2	3	2	1	3	1	2	3	311.32 ± 9.92
18	2	3	3	2	1	2	3	1	213.46 ± 0.71

S.D. = Standard deviation.

microbial metabolism²⁰. Therefore, statistical optimization of SSF factors for maximizing laccase production by *C. caperata* RCK2011 was attempted using eight critical fermentation factors and their selected levels (Table 1). These factors and their levels were selected based on our preliminary experiments on laccase production by *C. caperata* RCK2011.

Statistical optimization of laccase production. Effect of temperature, pH, wheat bran, inoculum size, CSL, KH₂PO₄, biotin and xylidine were studied by Taguchi method, which is a fractional factorial experimental design L₁₈ (2¹ × 3⁷) OA (Table 2). The results of experiments revealed that the maximum production of laccase (1576.13 Ugds⁻¹) occurred when experimental conditions were: temperature (30°C), pH (4.5), wheat bran (3 g), inoculum (0.5 ml), biotin - 0.4% (w/v), KH₂PO₄ - 0.03% (w/v), CSL - 0.1% (v/v) and xylidine - 0.5 mM. Whereas, minimum average production of laccase 49.23 Ugds⁻¹ was observed at temperature (35°C), pH (5.5), wheat bran (3 g), inoculum (1.5 ml), biotin - 0.5% (w/v), KH₂PO₄ - 0.07% (w/v), CSL - 0.1% (v/v) and xylidine - 1.0 mM.

Fermentation factors and their interactions on laccase production. The main effects of the fermentation factors at the assigned levels on laccase production are depicted in Table 3. By the term “main effects”, the average of obtained results (as unit of enzyme, produced per g of dry substrate), in which each factor is at given level, is meant. Production levels were found to be very much dependent on the culture conditions. Higher laccase production 865.0 and 861.66 Ugds⁻¹ were observed up to level 1 of pH and

Serial No.	Factor	Level 1	Level 2	Level 3	L2-L1
1	Temperature	861.666	501.666	-	-360.001
2	pH	865.0	830.5	299.5	15.5
3	Wheat bran	639.5	831.333	574.166	191.832
4	Inoculum	822.333	665.166	557.5	-157.167
5	Biotin	723	777.833	544.166	54.832
6	KH ₂ PO ₄	773.833	649.166	622	-124.667
7	Corn steep liquor	702.166	656.5	686.333	-45.667
8	Xylidine (inducer)	814.166	649	581.833	-165.167

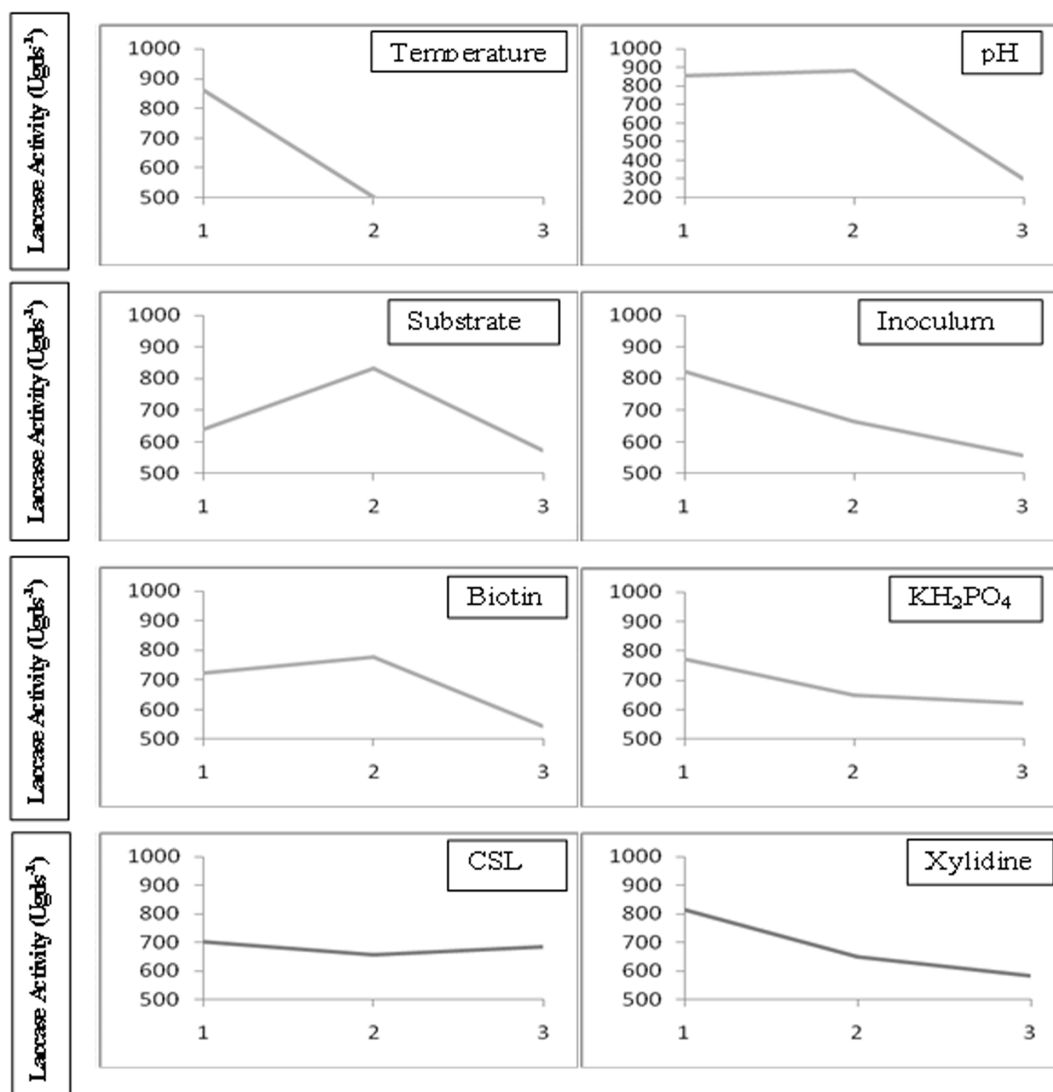


Figure 1 | Impact of selected factor levels on laccase production by *Coriolopsis caperata* RCK2011 under solid state fermentation.

temperature, respectively and above these values the enzyme production repressed. Wheat bran caused higher laccase production 831.33 Ugds^{-1} with increase in concentration at level 2 but not at level 3. Inoculum, KH_2PO_4 , CSL and xylidine had higher effects in level 1 on laccase production, whereas, increasing biotin concentration resulted in increase in laccase production 777.83 Ugds^{-1} up to level 2 and subsequent increase resulted in decrease in laccase production. The difference between the average value of each factor at level 2 and 1 (L2-L1) indicated the relative influence of the effect. The larger the difference the stronger the influence. The sign of the difference (+ or -) indicated whether the change from level 1 to level 2 or 3 increased or decreased the result. It can be seen from Table 3 that among the factors studied, wheat bran showed stronger influence (191.83 Ugds^{-1}) compared to other factors followed by biotin (54.83 Ugds^{-1}) and pH (15.5 Ugds^{-1}) in laccase production. Figure 1 represents the influence of each individual factor on laccase production.

Understanding the interaction between two factors gives a better insight into the overall process analysis. A factor may interact with any or all of the other factors creating the possibility of presence of a large number of interactions. The severity index (SI) was evaluated from Taguchi DOE that represents the influence of two individual factors at various levels of interaction. Table 4 shows the interactions of severity index for different factors. In this table, the 'Columns'

represents the locations to which the interacting factors are assigned. Interaction SI presents 100% of SI for 90° angle between the lines (factors), while 0% SI for parallel lines. If the interaction between the factors is reverse, that can be shown by 'Reserved column'. 'Levels' indicated the level of factors desirable for the optimum conditions. The highest interaction SI 85.94% was observed in between biotin and CSL (at levels 1 and 1; reserved column 2) followed by KH_2PO_4 and CSL (at levels 1 and 1; 1st reserved column) with SI 72.45%. It was interesting to note that biotin and CSL with less impact factor showed higher SI in combination. On the contrary, the SI interaction between high impact factors temperature and wheat bran was 11.4%. While, the least interaction SI 3.17% was found when strong impact factor pH interacting with wheat bran. It was evident from these observations that the influence of individual factors on laccase production had varying effects while in combination; the enzyme production was quite independent of the individual influence. Figure 2 shows the variation in laccase activity at chosen levels. Supplementary Figure S1 depicts the contribution of selected factors on the laccase production at optimum performance. pH has shown maximum positive impact on the production of laccase in individual cases. The data indicates relative interactions of factors on the laccase production.

Analysis of variance (ANOVA). Understanding the impact of each individual factor is the key for a successful fermentation process.



Table 4 | Estimated interaction of severity index for different factors

Serial No.	Factors	Columns	SI (%)	Reserved column	Levels
1	Biotin × CSL	5 × 7	85.94	2	[1,1]
2	KH ₂ PO ₄ × CSL	6 × 7	72.45	1	[1,1]
3	Biotin × KH ₂ PO ₄	5 × 6	69.76	3	[1,1]
4	CSL × xylidine	7 × 8	69.16	15	[1,1]
5	Wheat bran × inoculum	3 × 4	68.08	7	[1,1]
6	Inoculum × xylidine	4 × 8	60.37	12	[1,1]
7	Wheat bran × Biotin	3 × 5	53.99	6	[2,2]
8	KH ₂ PO ₄ × xylidine	6 × 8	53.2	14	[1,1]
9	Wheat bran × xylidine	3 × 8	51.82	11	[2,2]
10	Wheat bran × KH ₂ PO ₄	3 × 6	46.99	5	[1,1]
11	Inoculum × Biotin	4 × 5	46.1	1	[1,1]
12	Inoculum × KH ₂ PO ₄	4 × 6	40.6	2	[1,1]
13	Wheat bran × CSL	3 × 7	36.65	4	[2,1]
14	Temperature × xylidine	1 × 8	25.6	9	[1,1]
15	Temperature × CSL	1 × 7	25.32	6	[1,1]
16	pH × biotin	2 × 5	24.48	7	[1,1]
17	Inoculum × CSL	4 × 7	24.07	3	[1,3]
18	Temperature × pH	1 × 2	22.05	3	[1,3]
19	pH × xylidine	2 × 8	21.63	10	[1,2]
20	pH × KH ₂ PO ₄	2 × 6	20.86	4	[1,1]
21	Temperature × biotin	1 × 5	19.46	4	[1,2]
22	pH × CSL	2 × 7	12.19	5	[1,1]
23	Temperature × wheat bran	1 × 3	11.4	2	[1,2]
24	Temperature × inoculum	1 × 4	10.81	5	[1,1]
25	Biotin × xylidine	5 × 8	8.79	13	[1,1]
26	Temperature × KH ₂ PO ₄	1 × 6	7.79	7	[1,1]
27	pH × inoculum	2 × 4	5.61	6	[1,1]
28	pH × wheat bran	2 × 3	3.17	1	[1,2]

ANOVA was used to analyze the results of the OA experiment and to determine how much variation each factor has contributed (Table 5). From the calculated ratios (F) of all selected parameters, it was noticed that all factors and interactions considered in the experimental design were statistically significant at 95% confidence limit, indicating that nearly all the variability of experimental data can be explained in terms of significant effects. Statistical analysis of the laccase production data using above experimental designs revealed that among all selected factors pH contributed maximally (47%) on the overall enzyme production followed by the incubation temperature (21%). Wheat bran and inoculum concentration showed almost similar impact 7.76 and 7.70%, respectively on laccase production. Biotin and xylidine also showed impact of 6.41 and 6.21%, respectively. CSL and KH₂PO₄ showed the least impact (0.23 and 2.84%, respectively) at the individual level on overall production of laccase under the selected fermentation conditions, even though it has major impact on mass transfer of nutrients during microbial growth. This study revealed that overall 68.73% contribution was noticed with only two selected parameters

Table 5 | Analysis of Variance (ANOVA)

Serial No.	Factors	DOF (f)	Sums of squares (S)	Variance (V)	F-ratio (F)	Pure sum (S')	Percentage P (%)
1	Temperature	1	583200.223	583200.223	664004473.877	583200.223	21.119
2	pH	2	1315183	657591.5	1315183000	1315182.999	47.625
3	Wheat bran	2	214406.27	107203.135	2144062705.485	214406.27	7.764
4	Inoculum	2	212860.294	106430.147	2128602949.625	212860.294	7.708
5	Biotin	2	179176.276	89588.138	1791762762.858	179176.276	6.418
6	KH ₂ PO ₄	2	78666.302	39333.151	786663028.971	78666.302	2.843
7	Corn steep liquor	2	6452.337	3226.168	64523371.988	6452.337	0.233
8	Xylidine (inducer)	2	171540.39	85770.195	1715403900.553	171540.389	6.211
Other/error		2	-0.001	-0.001			0.004
Total		17	2761485.095				100.00

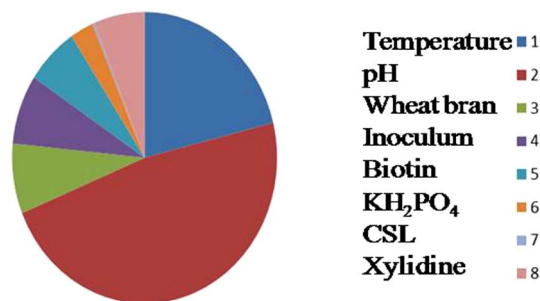


Figure 2 | Relative influence of factors and interactions.

(medium pH and incubation temperature) and rest 31.27% by other selected factors.

Optimum conditions and validation of laccase production. The Taguchi DOE provided optimum culture conditions for each factor and their contribution for achieving maximum laccase production. The optimum conditions and their contribution are shown in Table 6. Based on this information, optimum laccase production could be obtained at 30°C with medium (pH 5.0) consisting of 0.5% (w/v) biotin, 0.013% (w/v) KH₂PO₄, 0.1% (v/v) CSL, 0.5 mM inducer (xylidine) and 5 g wheat bran in 5 days of fermentation using 0.5 ml of inoculum. The pH and fermentation temperature have been found to play a significant role in the enzyme production compared to other selected factors. The expected laccase production at optimum conditions was 1692.16 Ugd^s⁻¹ with total contribution from all the factors being 1010.49 Ugd^s⁻¹ with grand average performance of 681.66 Ugd^s⁻¹. The observed 40% grand average performance of the fungal strain and 60% contribution of all fermentation factors revealed the potential of the fermentation factors concentration and their interaction for laccase production by the fungus, *C. caperata* RCK2011. It is evident from the observations (Supplementary Figure S2), that the optimized conditions enhanced laccase production of 59.71% i.e. from 681.66 to 1692.16 Ugd^s⁻¹. Further to validate the proposed methodology, experiments were performed for laccase production by employing the optimized culture conditions. The validation resulted in the laccase production of 1623.55 Ugd^s⁻¹ (expected response 1692.16 Ugd^s⁻¹), thus proving the validity of the method.

Discussion

Among statistical methods mostly Plakett-Burman design and RSM are preferred for optimizing biotechnological processes, could be because researchers are more familiar with them^{8,21,22}. However, there are few reports showing usefulness of Taguchi methods in optimization of biotechnological process have appeared¹⁶. This method may be used quite easily and is less cumbersome. This method also has the ability to include physical factors along with



Table 6 | Optimum culture conditions and their contribution

Serial No.	Factors	Values	Level	Contribution
1	Temperature (°C)	30	1	180
2	pH	5.0	2	198.833
3	Wheat bran (g)	5.0	2	149.666
4	Inoculum (ml)	0.5	1	140.666
5	Biotin (% w/v)	0.5	2	96.166
6	KH ₂ PO ₄ (% w/v)	0.013	1	92.166
7	Corn steep liquor (% v/v)	0.1	1	20.5
8	Xylidine (inducer) (mM)	0.5	1	132.5

Total contributions from all factors = 1010.497.

Current grand average performance = 681.666.

Expected result at optimum conditions = 1692.163.

the nutritional factors in the shortened fractional factorial designs. After primary studies of culture conditions, the laccase production was raised to 77% indicating the importance of fermentation factors in enzyme production. The enzyme secretion depends on the physiological, nutritional and biochemical nature of the microorganism employed, and even on the strain of the microorganism (s)^{6,23,24}. The statistical optimization with the Taguchi L₁₈ OA showed a significant variation in enzyme production ranging from 49.23 to 1576.13 Ugds⁻¹ (Table 2). Similar variation of enzyme production with optimization experiments were noticed for laccase production by *Pleurotus ostreatus* 1804²⁰. Production levels were found to be very much dependent on the culture conditions (Table 3). pH and temperature effectively produced maximum laccase (865.0 and 861.66 Ugds⁻¹) at level 1, whereas, wheat bran and biotin produced maximum laccase (831.33 and 777.83 Ugds⁻¹) at level 2. It was observed that wheat bran showed stronger influence (191.83 Ugds⁻¹) compared to other eight factors in the laccase production. It is reported that wheat bran is the good substrate for the growth and its soluble cellulose and hemicelluloses fractions served as carbon source which leads to a sufficient carbon and nitrogen ratio for efficient laccase production²⁵. Wheat bran is used as a lignocellulosic source, which provides carbon and nitrogen source to the microbes. Wheat bran was reported as excellent growth substrates for laccase production by *Corioliopsis unicolor*²⁶. The type and amount of the lignolytic enzymes produced by the white rot fungi depends on the type of the plant material as substrate.

The interaction between biotin and CSL with less impact factor showed higher severity index (85.94%). CSL supplemented as organic N₂ source in the medium has been reported to increase laccase production by *P. ostreatus*²⁷. CSL is a complex substrate produced in the corn-processing industry and composed of peptides, sugars, lactic acids, vitamins and metallic ions²⁸. CSL supplies nitrogen and carbon sources, and improves fungal growth during initial stages of colonization. The supplementation of biotin stimulated laccase production by *C. bulleri*. The increase in enzyme production could possibly be due to the alteration of membrane permeability, exocytosis, hyphal sheath desorption, enzyme stabilization, or even due to structural changes of the enzyme molecule itself⁵. The interaction between KH₂PO₄ and CSL produced second highest SI 72.45%. The elemental phosphorous is essential to living organisms because it is the part of the backbone of DNA, the carrier and transmitter of genetic information in cells. The optimum phosphorous content not only supported robust growth, but also helped in production of higher MnP, another lignin degrading enzyme, by *Phanerochaete chrysosporium*⁷. Moreover, CSL revealed varied interaction SI values with other factors such as xylidine (69.16%), wheat bran (36.65%), temperature (25.32%), inoculum (24.07%) and the least 12.19% with pH (strong impact factor).

Xylidine is the most widely reported inducer of laccase production^{29–31} and enhanced laccase specific production by 4 folds

in *Corioliopsis polyzona*³². The aromatic compounds accelerated the enzyme production and shortened the time of peak activity appearance.

Laccase production increased with increasing inoculum concentration up to a critical value beyond which it decreased. Lower level of an inoculum may not be sufficient enough for initiating the growth of the organism, whereas, higher level may cause competitive inhibition³³. However, the interaction among the strongest impact factor temperature and pH depicted SI 22.05%. Temperature is one of the most important factors affecting SSF and the optimal temperature generally lies in the range of 25–30°C³⁴. The pH of the medium is also one of the most detrimental environmental factors affecting the mycelial growth, enzyme production and the transport of various components across the cell membrane³⁵.

ANOVA analysis presented that pH contributed maximum impact (47%) followed by temperature, wheat bran, inoculum, biotin and xylidine (Table 5). While KH₂PO₄ and CSL showed the least impact factor 2.84 and 0.23%, respectively. The observed percent effect of the CSL and KH₂PO₄ on overall laccase production in the present investigation should be viewed with caution when compared to that of SSF with a defined medium composition. This is mainly due to the effect of a solid substrate (wheat bran) that provides support for microbial growth and also its role as carbon, nitrogen, vitamins, and proteins sources. The higher production with wheat bran is consistent with earlier reports on SSF using wheat bran as substrate^{36–38}.

The present study revealed that medium pH and incubation temperature contributed 68.73% and rest 31.27% by other selected factors (Table 5). It clearly indicated that physical factor - pH and temperature are vital factors for laccase production by *C. caperata* RCK2011 under SSF. The validation of optimum conditions derived from Taguchi optimization process showed an increase of 58.01% in laccase production (Supplementary Figure 2). Similarly, Taguchi DOE methodology has been applied for optimizing laccase production in *P. ostreatus* 1804²⁰ and *P. ostreatus* IMI 395545³⁹ under submerged fermentation.

In conclusion, the application of Taguchi DOE helped in reaching the optimal solutions and in critically analyzing the interactive effects of most influential parameters on laccase production by *C. caperata* RCK2011 for better understanding the bioprocess for improving the enzyme productivity.

Methods

White-rot fungus. *Corioliopsis caperata* RCK2011 a fungus isolated from the Aravali range forest in University of Delhi South Campus (UDSC), New Delhi, India, was grown and maintained on Malt Extract Agar (MEA) containing (g/L): Malt extract 20.0, KH₂PO₄ 0.5, MgSO₄·7H₂O 0.5, Ca (NO₃)₂·4H₂O 0.5, Agar 20.0 (pH 5.0) at 30°C^{5,40} and stored at 4°C.

Each 250 ml Erlenmeyer flask containing 50 ml of Malt Extract Broth (MEB) consisted of (g/L): Malt extract 20.0, KH₂PO₄ 0.5, MgSO₄·7H₂O 0.5, Ca (NO₃)₂·4H₂O 0.5 (pH 5.0) was inoculated with four mycelial discs (8.0 mm diameter each) and incubated at 30°C under static cultivation conditions for 7 days. The fungal mats thus obtained were homogenized using pestle and mortar under sterile conditions and were used as inoculum in solid state fermentation experiments.

Taguchi methodology. Optimization methodology adopted in this study was divided into four phases viz., planning, experimentation, software analysis, and validation of results. Each phase has separate objective and is interconnected in sequence to achieve the overall optimization process.

Design of experiments (phase I). Taguchi DOE was used to set up the critical fermentation factors such as temperature, pH, wheat bran, inoculum size, CSL, KH₂PO₄, biotin, xylidine (an inducer) that have a significant influence on laccase production. The copper is generally used to enhance laccase production as it regulates the laccase gene transcription in the gene fermentation of the fungi. During optimization for laccase production following one factor at a time method, copper increased the laccase production by 17%, while xylidine induced laccase production by 38%. Therefore, xylidine was selected instead copper as an inducing factor. In the next step, matrix was designed with the appropriate OA for the selected factors and their levels. In the present study, OA L₁₈ (which indicated 18 experimental trials) was selected for above controlled factors with three levels of factor variation (Table 1). All



these factors were assigned with three levels, except incubation temperature which was assigned with two levels with a layout of $L_{18} (2^1 \times 3^7)$.

Solid state fermentation (phase II). Solid state fermentation (SSF) experiments were performed for laccase production with *C. caperata* RCK2011 employing the selected 18 experimental trails (Table 2) in combination with eight factors at selected levels (Table 1). Prior to use, wheat bran was washed and dried in an oven (50°C; 24 h). All experiments were conducted in 250 ml Erlenmeyer flasks, each containing wheat bran (3, 5 or 7 g) with relative moisture content (in 1:3 ratio) containing NH_4Cl (0.15% N_2 equivalent), MgSO_4 (0.1% w/v) and varying amount of KH_2PO_4 (0.03, 0.05 or 0.07% w/v), CSL (0.1, 0.2 or 0.3% v/v), dissolved in distilled water, the pH of mineral salt solution was adjusted to 4.5, 5.0 or 5.5 using 0.1 N NaOH or 0.1 N HCl. Flasks were sterilized by autoclaving at 121°C (15 psi) for 15 min. The flasks were inoculated with selected levels of homogenized inoculum 0.5, 1.0 or 1.5 ml (0.0075, 0.015 or 0.0225 g dry wt.) and incubated at different temperatures (30 or 35°C) in an incubator for 5 days. After 48 hrs of inoculation, the flasks were supplemented with filter sterilized biotin (0.4, 0.5 or 0.6% w/v) and xylinidine (0.5, 1.0 or 1.5 mM). The entire contents of the flask were used for enzyme extraction and estimation. The laccase activities presented in this study are the average of three individual determinations.

Analysis of experimental data and prediction of performance (phase III). The influence of individual factor on laccase production and their performance at optimum conditions using Taguchi approach were analyzed by Qualitek-4 software (Nutek Inc., MI, USA). The software is equipped to use from L-4 to L-64 arrays along with selected 2-63 factors and their 2-4 levels. The automatic design option allows Qualitek-4 to select the array used and assign factors to the appropriate columns.

Validation (phase IV). In order to validate the optimized methodology, the SSF fermentation experiments were performed in triplicates for laccase production using the optimized culture conditions.

Extraction and estimation of laccase. The fermented wheat bran from each flask was suspended in citrate phosphate buffer (100 mM, pH 5.0) in 1:10 ratio (w/v) and shaken gently for 45 min. The extrudates were squeezed through muslin cloth for maximizing the enzyme extraction and centrifuged at 6708 g for 20 min at 4°C. The enzyme solution thus obtained was assayed for laccase activity. Laccase (EC 1.10.3.2) activity was measured using Guaiacol as a substrate^{29,41,42}. The reaction mixture (1 ml) contained 0.1 ml of the enzyme extract and 0.9 ml Guaiacol (10 mM) prepared in 100 mM citrate phosphate buffer (pH 5.0). The molar extinction coefficient of tetraguaiacol at 470 nm ($26.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) was used for activity calculation⁴³. One unit of laccase was defined as the amount of the enzyme required to transform 1 μmol guaiacol/min. The laccase activity is expressed as unit per gram of dry substrate (Ugds^{-1}).

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Author contributions

R.C.K. conceptualized the study. R.S.R. designed the statistical experiments. P.N. performed experiments, R.C.K. and P.N. analyzed the results. R.C.K., P.N. and R.S.R. prepared the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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